11. USING STEM CELLS TO BUILD NEW BONES: A TISSUE ENGINEERING FRONTIER

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INTRODUCTION: BONE STRUCTURE, FUNCTION, AND FORMATION

Bone, the primary component of the skeleton, helps us perform a variety of mechanical functions such as support, movement, and protection of internal organs in addition to providing the home for blood formation (hematopoiesis) and regulating certain metabolic functions. Like cartilage and adipose (fat) tissue, bone is a connective tissue, meaning that it contains a population of cells that are embedded into an extracellular matrix.[1] Bone and related structures found in teeth are unique among connective tissues in that their matrices include elements that make them rigid (crystalline mineral, hydroxyapatite) as well as elastic elements (type I collagen). In the correct proportion, these elements allow bone to bear the mechanical stresses imposed by daily life; the tissue is harder than cartilage yet sufficiently flexible not to be brittle. However, an imbalance in the matrix composition can contribute to diseases. For example, a deficiency in the quantity or quality of collagen produced can cause osteogenesis imperfecta, a form of brittle bone disease. Furthermore, correct orientation of matrix elements is also necessary to confer bone strength. Newly-deposited bone, such as that of infants or that formed immediately following a fracture, features a mechanically-weak "weave" of collagen fibers. As bone formation continues, this woven bone is replaced by sheets of bone (lamellae) capable of withstanding the stresses required for normal activity.

In healthy individuals, bone regenerates through a two-step remodeling process that involves resorption of mature bone that has become fatigued and the formation of new bone tissue (ossification) (See Fig. 11.1 on the next page). Osteoclasts bring about resorption by breaking down the bone and releasing minerals into the bloodstream. Ossification is facilitated by osteoblasts, mature bone cells that secrete the protein mixture that mineralizes to become bone. These synchronized processes occur at low levels throughout life to promote turnover and maintain tissue health, and they are essential to shape and integrate new bone tissue following fracture or during growth. Disruptions to the processes can lead to the net loss of bone density characteristic of common conditions such as osteoporosis. An estimated 40 million persons in the United States currently either have or are at high risk for osteoporosis,[2] indicating a need for strategies to promote new bone growth.

A supply of healthy bone tissue therefore depends on a dynamic balance of elements, and the formation of viable new bone thus requires appropriate structure and timing. Bone's unique and well-studied combination of structural properties has led researchers to apply engineering principles to help the body construct new bone to replace that lost through disease or decay. Because bone marrow stromal cells (BMSCs) contain a subset of stem cells (also called mesenchymal stem cells, multipotent stromal cells, or skeletal stem cells) that can differentiate into osteoblasts, these stem cells play a vital role in the "tissue engineering" of new bone. This article will highlight research on the use of BMSCs to provide the body with a means to regenerate lost bone tissue and will discuss the challenges and possible ramifications of using such strategies in the clinic.

TISSUE ENGINEERING: STEM CELLS AND SCAFFOLDS

"Tissue engineering" encompasses a number of approaches to restore lost or damaged tissue. In some cases, the direct introduction of cells alone may be sufficient to regenerate tissue and/or restore function. For example, stem cells have been long been used in bone marrow transplantation, and limited studies have suggested that stem cells injected directly into damaged cardiac tissue can restore some function (see chapter, "Mending a Broken Heart: Stem Cells and Cardiac Repair," for more details). However, most

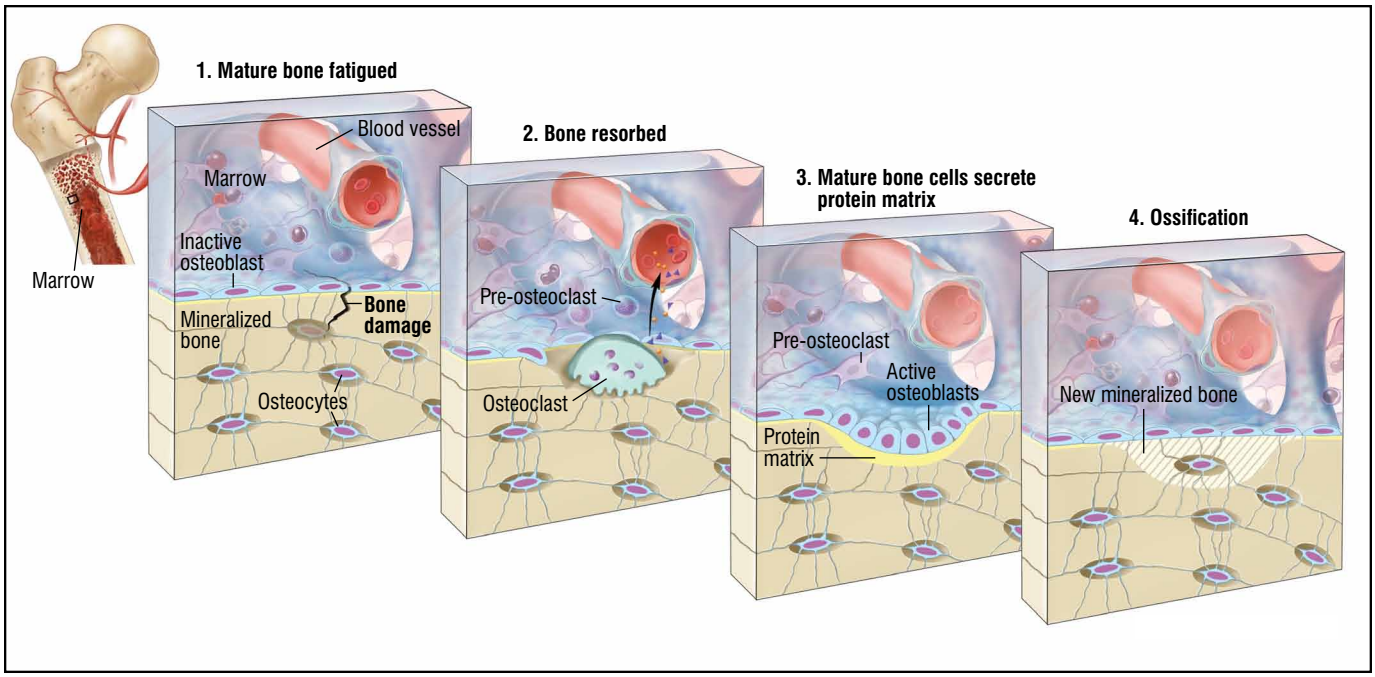

Figure 11.1. Repair of bone.

1. Mature bone becomes weakened and/or damaged. Osteocytes adjacent to the weakened or damaged bone die.
2. Remodeling is stimulated by the bone damage. Pre-osteoclasts from the bone marrow mature into osteoclasts and resorb the damaged bone.
3. Pre-osteoblasts (found surrounding blood vessels and within the bone marrow) are stimulated to mature into active osteoblasts. Active osteoblasts secrete protein matrix to replace the resorbed bone.
4. The non-mineralized matrix is hardened into new mineralized bone – a process called ossification. Active osteoblasts are called osteocytes after they become entrapped in their own secreted matrix.

Bone growth and modeling follow similar steps to this bone repair process. Unlike bone damage and subsequent remodeling, however, growth and modeling are stimulated by hormones, stress from muscular contractions and movement, and growth factors. In order for a hardened bone to grow, the body must break down some of the bone while simultaneously producing new bone to add to its length. During growth and modeling, the rate of bone deposition is greater than the rate of resorption.

tissue engineering applications require a combination of materials, specifically chosen to meet cellular, molecular, and mechanical requirements. For example, the formation of new bone requires an established paradigm that introduces populations of BMSCs that contain stem cells in the presence of a biomaterial "scaffold." In this context, a scaffold is an implantable material, either natural or synthetic, that provides appropriate support to a developing tissue.[3] The scaffold can be thought of as a delivery vehicle for a seed population that contains stem cells and as a structural support for the burgeoning tissue that forms as the cells differentiate. To encourage tissue formation, the stem cells are attached to the scaffold and transplanted immediately, sometimes in conjunction with appropriate tissue growth factors. Choice of scaffolding depends on application, as materials must be compatible with the stem cell population and the tissue that is being regenerated. Ideally, a scaffold will: 1) enable the stem cells to retain critical characteristics such as the ability to self-renew; 2) allow the cells to differentiate appropriately; 3) provide adequate support for the developing tissue; 4) conform to the mechanical specifications of the injury site; and 5) ultimately be resorbed into the body without generating toxic by-products.[3,4]

Researchers have investigated a wide variety of natural and synthetic materials and composite mixtures of materials as scaffolds for bone regeneration. Natural proteins such as collagen,[5-7] fibrin,[8] silk,[9-11] and polysaccharides (chains of sugar molecules) such as hyaluronic acid,[12-14] and chitosan[15] have demonstrated potential as components of bone scaffolding. These materials offer the advantages of biocompatibility, biodegradability, limited toxicity, and the ability to

be molded to meet the mechanical requirements of bone.[3,8] However, purity issues and a limited range of mechanical properties of these materials used by themselves have led researchers to use these materials in composite mixtures with substances such as hydroxyapatite, calcium phosphate, or silica to facilitate a wider range of cell behavior. Synthetic biomaterials, including those based on polymers,[16-18] peptides,[19] and ceramics[20,21] have also been explored as structural supports for bone scaffolding. These materials offer superior reproducibility relative to natural materials; their defined chemical composition enables independent control of mechanical properties, degradation rate, and shape.[3] However, biocompatibility becomes a concern, as these materials may need to be modified to help cells bind to them and may trigger immune responses in recipients.[3]

Materials development for tissue engineering is a rapidly-expanding area of research interest, and investigators are reporting improvements in scaffold design and delivery. Successful formation of complex tissue *in vivo* requires the concerted action of numerous processes, and scaffold design is becoming more sophisticated to capture the cellular and molecular nuances of bone formation. In addition, researchers have begun to develop hybrid systems that use nanoparticles to help transport these biomaterial scaffolds and cells to the sites of action.[22-24] While researchers routinely test these materials in animal models, several challenges remain before they can be used in the clinic (see below).

THE BONE MARROW-DERIVED MESENCHYMAL STEM CELL POPULATION AS A RESOURCE FOR REGENERATING BONE

In 1966, A.J. Friedenstein and colleagues at the Gamaleya Institute in Moscow isolated a population of cells from mature mouse bone marrow that formed new bone when re-transplanted into an adult mouse.[25] These cells, which Friedenstein called "colony forming unit-fibroblasts" have since been termed "mesencyhmal stem cells," the name by which they are most commonly known. The cells' ability to adhere to the plastic culture dishes used in the experiment facilitated their initial isolation and has enabled researchers to carry out numerous manipulations in subsequent experiments. Given their discovery more than 45 years ago, MSCs are among the longest-studied of all stem cell populations, and most osteogenic tissue engineering strategies use MSCs as the seeding cell population. MSCs, which have since been isolated in human bone marrow, are multipotent, with demonstrated ability to differentiate *in vitro* into a wide range of cell types, including osteoblasts, cartilage cells (chondrocytes), and fat cells (adipocytes).[26-28] MSC-like cells have also been identified in tissues such as fat, dental pulp, tendons, umbilical cord blood, and skeletal muscle, although it is not known whether these cell populations are equivalent to those found in bone.[29]

However, the exact composition of the bone marrow's resident stem cell population remains a bit of a mystery. Marrow clearly contains a heterogeneous population of non-hematopoietic stem or stem-like cells, some of which are multipotent. MSCs represent a subset of this larger population, although ascertaining their true composition *in vivo* is challenging. Nearly all current understanding of the properties of MSCs has been generated from studying cultured cells. Relatively little is known at present about their origin, location, and behavior in living systems,[29] although recent studies demonstrate that MSCs are pericytes; that is, cells associated with the walls of marrow sinusoids.[30] Moreover, researchers have yet to identify specific cell markers that characterize MSCs definitively *in vivo,* thereby hampering the ability to develop a rigorous assay to test their self-renewal potential beyond the culture dish.[29]

Although the heterogeneity of MSC populations has challenged efforts to map behavior *in vivo* to specific cell types, this diversity enables MSCs to support numerous potential applications in the clinic (see below for examples). Interestingly, many studies with MSCs suggest that the cells engraft with a surprisingly low frequency given their therapeutic efficacy.[28] Part of the efficacy of these cells may lie in the MSC populations' ability to secrete a variety of soluble molecules that affect processes associated with healing, such as inflammation and angiogenesis,[28,31] or to recruit to the area other cells implicated in tissue regeneration.[28,32]

CLINICAL APPLICATIONS AND CHALLENGES

MSCs' "multipurpose" capability makes these cells a potential resource for many tissue regeneration and engineering applications. In principle, MSCs could support autologous transplantations; i.e., procedures in which a patient's marrow is harvested for stem cells that are then expanded in culture and reintroduced into the

patient under conditions that promote differentiation. This well-established regeneration strategy serves as the basis for bone marrow transplants to treat leukemia and other blood-based diseases, in which a patient's hematopoietic (blood-forming) stem cells are harvested from the blood or bone marrow and reintroduced following chemotherapy or radiation designed to kill the malignant cells in circulation. Using one's own stem cell population minimizes the chance of graft-versus-host disease, an immune response induced by the presence of the reintroduced cells. If MSCs prove amenable to such an approach, then the cells could theoretically apply to a wide range of diseases so long as the differentiation and delivery processes could be controlled with the aid of prudently designed biomaterial scaffolds.

MSCs have demonstrated therapeutic efficacy in animal models of diseases ranging from diabetes[33] and kidney disease[34] to lung injury[35] and myocardial infarct.[36] In these experiments, MSCs were isolated from human or rodent bone marrow, cultured *in vitro,* and usually applied systemically, through injection into the jugular vein or heart. As of 2010, more than 50 clinical trials related to MSCs have also been reported.[37] Many of these use allogeneic MSCs (e.g., MSCs that come from a matched or unmatched donor) as a post-transplantation therapy to enhance the success of bone marrow transplantation to treat conditions such as osteogenesis imperfecta,[38] metachromatic leukodystrophy and Hurler syndrome,[39] and severe graft-versus-host-disease.[40] The osteogenesis imperfecta study demonstrated the powerful potential of these cells to create new bone, as improvements were noted in five of the six patients despite the fact that less than 1% the donor MSCs appeared to be engrafted.[38] A recent study has demonstrated that human fetal and adult MSCs delivered in polymeric scaffolds can repair large bone defects in the rat.[41] Furthermore, limited studies have also shown that autologous MSCs seeded into shaped biomaterial scaffolds can be implanted to replace[42] or repair[43] human bone defects.

Despite these promising reports, however, the use of human MSCs to repair or regenerate bone is currently in a pre-clinical stage. Several challenges must be overcome before these cell populations are ready for clinical tissue engineering applications. Although researchers have demonstrated that MSCs can be isolated and cultured using a variety of approaches, no single method is considered standard,[28] and comparative studies are rarely reported.[41] Using various methods to isolate MSC populations, researchers have identified common cell-surface protein markers, some of which could correlate with differentiation lineages. However, some isolation schemes introduce genetic and epigenetic changes in the cells that may affect their therapeutic capabilities. Moreover, genetic variation among bone marrow donors can affect the biological activity of MSC populations even when the isolation conditions are held constant.[28]

The basic process for transplanting MSCs to regenerate bone includes isolating the cells from the patient, culturing them to expand the cell number *in vitro,* seeding them onto scaffolds, and implanting the scaffold appropriately.[44] Each of these steps must be carried out under strict quality control to minimize the introduction of unwanted by-products. For example, animal-based components in the culture medium can introduce foreign proteins into the cultured cells that subsequently heighten the immune response even if the cells themselves are allogeneic.[37] Cultured MSCs represent a complex, heterogeneous mixture of stem/progenitor cells and more mature cells.[37] Although it would be ideal to purify the exact populations of cells that will be most useful for a given application, the inability to do so does not appear to be a critical limitation when using MSCs.[4] However, the safety of these cells, especially when delivered in conjunction with a scaffold, has not been fully explored in clinical trials.

Selecting culture conditions that maintain the appropriate stem cells within the population is paramount for MSC-based therapy to be efficacious. Various physical, chemical, and biological cues in the stem cell microenvironment guide differentiation *in vivo,* and researchers have begun to identify molecular signaling pathways that govern bone MSC proliferation and maintenance[45] and physical growth constraints that influence differentiation of MSC cultures.[46] For instance, MSCs grown in a star shape, which promotes a tense cytoskeleton, become bone cells, whereas most MSCs grown in a flower shape, which promotes a loose cytoskeleton, become fat cells.[46] Understanding such subtleties will enable researchers to optimize the conditions under which these cells can be coaxed into creating new bone when implanted.

Finally, the cell/scaffold complex must be delivered appropriately (either locally or systemically) to assure that the construct reaches its target and that the cells

remain viable once there. As with other potential stem cell-based therapies, researchers must be able to track injected cells to measure the success of therapy and to assess the potential for side-effects or other unintended consequences. Although the heterogeneity of MSC populations challenges researchers' efforts to identify a small, definitive set of cellular "tracking" markers, implanting the cells in a scaffold opens up opportunities to track the cells using nanoparticles or other deliverables.[41] Most of these challenges are common to all putative stem cell-based therapies, and overcoming them will ultimately require knowledge of the biology of the cells and how their behavior is influenced by context. The construction of new bone is but one application of these powerful stem cells; harnessing their power will point scientists toward a range of therapeutic applications.

CONCLUSION

Establishing new bone tissue in response to disease or injury involves a delicate balance of molecular processes and the coordination among various cell types. As with other regenerative medicine applications, researchers are exploring ways to utilize stem cells to engineer new bone tissue. While MSCs represent an obvious choice because they can differentiate into osteoblasts, preliminary experiments have demonstrated that this heterogeneous, marrow-derived cell population promotes a range of cell behaviors beneficial for regenerating tissue. When delivered in conjunction with a biomaterial scaffold, MSCs have demonstrated the ability to help establish new bone in animal models. This strategy offers researchers the opportunity to tailor scaffold construction and content to deliver the cells appropriately, track their location, and encourage appropriate differentiation. As scientists accrue knowledge of MSC biology, cell culture conditions and protocols will become more standardized, thereby enabling the comparative studies necessary for clinical development.

Seeding scaffolds with stem cells to regenerate bone demonstrates how scientists and engineers collaborate to address a clinical challenge. This confluence of disciplines reflects the complexity of regenerative medicine and the vast potential of tissue engineering. While the optimal combinations of culture conditions and scaffold design have yet to be defined, tissue engineering with stem cells will continue to be a central paradigm for repairing damaged bone and other tissues, thus illuminating another frontier of future therapy.

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